

Major Histocompatibility Complex Haplotypes and Complement C4 Alleles in Systemic Lupus Erythematosus

Results of a Multicenter Study

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Abstract

In a multicenter study more than 300 central European systemic lupus erythematosus (SLE) patients were examined for HLA-B, HLA-DR, and complement C4 phenotypes. For 174 SLE patients MHC haplotypes were determined by family segregation analysis, and for 155 patients C4 gene deletions were determined by TaqI restriction fragment length polymorphism. Two haplotypes, B8-C4AQ0-C4B1-DR3 and B7-C4A3-C4B1-DR2, were identified as risk factors for SLE. These findings were confirmed by applying the haplotype frequency difference (HFD) method, which uses nontransmitted haplotypes from the family study as internal controls. Furthermore, only HLA-DR2, but not DR3, B7, or B8, was significantly increased in SLE patients independently of the two risk haplotypes. C4A gene deletions, but not silent C4AQ0 alleles, were increased in SLE patients and neither C4BQ0 alleles nor C4B gene deletions were increased. The observed frequencies of homozygosity and heterozygosity for the two haplotypes and the frequencies of homozygotes for C4AQ0 and C4A deletions did not differ from the expected values, indicating that the risk for SLE is conveyed by single allele effects. In conclusion, there are two MHC-linked susceptibility factors for Caucasian SLE patients carried by the haplotypes B7-DR2 and B8-DR3. The results argue against C4Q0 alleles being the decisive factors increasing susceptibility to SLE. (*J. Clin. Invest.* 1992. 90:1346-1351.)
Key words: systemic lupus erythematosus • genetics • HLA • family studies • autoimmunity

Introduction

The etiology of systemic lupus erythematosus (SLE) is unknown. The importance of genetic factors has been shown through family studies in identical twins and first degree relatives, but the contribution of various immunogenetic factors is still controversial (1). Initial reports of immunogenetic associa-

tions with inherited CR1 (2) and FcR defects (3), acetylator phenotype (4), immunoglobulin allotypes (5), and Ti-polymorphism (6) have been found to be acquired (7), nonexistent (8, 9), or still highly controversial (10, 11). Like numerous other diseases, SLE is associated with genes of the MHC, but it is not clear which genes are primarily responsible for the association. In Caucasians, SLE is associated with the presence of the HLA antigens B8, DR3, and DR2, and with C4AQ0, but not C4BQ0 alleles (12), although many previous reports examining smaller numbers of patients have provided other results: some groups found an increase in DR2 but not DR3 (13-15), others found DR3 elevated, but not DR2 (16, 17), and some investigators reported an increase of C4BQ0 in SLE (18, 19). The components of the MHC haplotypes that are responsible for these associations, and the pathomechanisms involved, are still a matter of speculation. Presently, two main theories prevail. The first theory assumes that C4Q0 alleles are the decisive factors for MHC associations (20, 21); the second postulates that MHC class II molecules (DR, DQ) are involved in susceptibility to SLE and other autoimmune diseases (22-24). To clarify which MHC alleles are primary risk factors for SLE and to address the question whether or not the Q0 alleles of C4 are the main predisposing factors for disease, we initiated a multicenter family study of a large number of patients. We have determined the HLA antigens and analyzed C4A and C4B by high voltage agarose gel electrophoresis (25), α -chain quantitation (26), and TaqI restriction fragment length polymorphism (RFLP)¹ (27) in more than 300 SLE patients and have determined MHC haplotypes by segregation analysis in 174 SLE families.

Methods

Patients. 417 Caucasian SLE patients of central European descent were recruited from the outpatient clinics of four centers (Hannover, Freiburg, Erlangen, and Düsseldorf) between 1986 and 1990. Overlap syndromes were carefully excluded and all patients fulfilled the revised American Rheumatism Association criteria for SLE (28). All patients were invited to participate in a family study. Of 192 SLE patients, various first degree relatives and spouses provided samples for HLA and C4 typing. In 174 cases the HLA-B-C4A-C4B-DR haplotypes of SLE patients could be unequivocally determined by segregation analysis.

HLA typing. HLA typing of A, B, DR, and DQ antigens was performed using a standard complement-dependent microcytotoxicity as-

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1. *Abbreviations used in this paper:* RFLP, restriction fragment length polymorphism; RPE, relative predispositional effects.

say (29). HLA-A, B, DR, and DQ typing for the patients from Hannover, Freiburg, and Düsseldorf was performed in the HLA typing laboratory of the Medical School Bloodbank at Hannover. Individuals from Erlangen were HLA typed in the HLA typing laboratory of the Department of Immunology in Erlangen. As external controls, HLA and two-point haplotype frequency data from 1,900 European Caucasian haplotypes of the 1984 HLA Workshop (30, 31) and from 975 healthy German controls (32) were used, and an internal control group of 265 haplotypes was obtained using those haplotypes not transmitted from family members to the individual with SLE (33) as well as the haplotypes of the spouses of the SLE patients (consequently termed "non-SLE haplotypes"). This group represents an ideal control sample, since its members originate from the same geographic and genetic population as the 174 SLE patients included in the family study.

Phenotyping of C4. C4 allotypes were determined from neuraminidase-treated EDTA plasma samples using high voltage agarose gel electrophoresis followed by immunofixation, as previously described (25). In 80% of all patients and relatives, C4- α -chains were quantitated by SDS-PAGE, thereby defining C4 null alleles based on the relative amounts of C4A and C4B gene products (26).

TaqI RFLP of C4 and 21-hydroxylase genes. Of 155 SLE patients and 235 relatives and spouses, genomic DNA was digested with the restriction enzyme TaqI and hybridized with ³²P-labeled 5' C4 and steroid 21-hydroxylase (CYP21; 21-OH) probes to study the C4/21-OH gene structures. The 6.4-kb C4 fragment is in most cases a marker for a deletion of an entire C4A gene together with the 3' adjacent 21-OHA pseudogene, which is usually present in the complotype C2C-BfS-C4AQO-C4B1, as part of the extended haplotype Cw7-B8-DR3. Furthermore, two different types of C4B gene deletions, one with deleted C4B/21-OHB genes and one with deleted 21-OHA/C4B genes, were detected. For a detailed description of the methods and interpretation of restriction fragments see (27, 34).

Family study. From a total of 417 SLE patients, complete HLA-B, DR, and C4A, C4B phenotypes were obtained in 343 SLE patients. Relatives and spouses of 192 patients volunteered for the family study and were analyzed for HLA-A, B, DR, and DQ antigens, and C4A and C4B allotypes.

Altogether, 453 first degree relatives (parents, siblings, and children) and spouses were examined and a definite assignment of HLA haplotypes by segregation analysis was achieved for 174 SLE patients and their relatives. This group is thereafter referred to as the "family study." TaqI RFLP was performed in 155 of 343 SLE patients, including 100 patients from the family study and 235 relatives and spouses.

Four patients with familial SLE in two families were not included in the study. All spouses and relatives were interrogated for SLE and its symptoms. 254 spouses and relatives were tested for antinuclear antibodies (by HEP-2 immunofluorescence) and 388 for dsDNS antibodies (by ELISA). Only 2 of 388 relatives had low titer antibodies against dsDNS, but no clinical SLE. Of 254 patients, only 12 had antinuclear antibody titers of over 1:160 (nine times 1:320, three times 1:640), but none of these had clinical signs of SLE, and they were therefore not removed from the internal control population.

From the family study (174 patients), a total of 265 haplotypes, including those present in the spouses of SLE patients or the parental haplotypes which were not transmitted to the SLE patients, were defined by segregation analysis. For 155 of these haplotypes TaqI RFLP data was obtained.

For statistical analysis the chi-square test was used and uncorrected *P* values are given unless otherwise indicated. Relative predispositional effects (RPE) were assessed by sequential removal of alleles showing the largest deviation from the expected number of patients according to the control group (35). Relative risks were calculated from phenotype frequencies as described in reference 36.

Results

In over 350 SLE patients phenotyped for HLA-B, HLA-DR, C4A, and C4B, the HLA alleles DR2 and DR3 were significantly increased over controls (Table I a). The removal of the DR2 and DR3 alleles (Table I b) makes evident that there is no significant protective (or susceptibility) effect of any other DR allele. The C4AQ0 allele but not the C4BQ0 allele was increased in SLE (Table II). HLA-B8, but not B7 or any other HLA-B antigen, was significantly increased in SLE. The gene frequency of B8 in 390 SLE patients was 24.0% versus 9.6% ($P < 10^{-8}$) in controls (31) and of B7 was 14.5% in SLE versus 11.5% in controls ($P = 0.09$; not significant). HLA-B-DR haplotype counting in 174 SLE families showed that the frequencies of two haplotypes, B7-DR2 and B8-DR3, were significantly increased over the controls (Tables III and IV). The frequencies of all other haplotypes did not differ significantly from the control samples (data not shown). These observations were confirmed by haplotype estimation using the haplo-

Table I. HLA-DR Gene Frequencies (a) and RPE (b) of HLA-DR Alleles in SLE (Control from [31])

HLA	SLE (n = 712 alleles)	Controls (n = 1,950 alleles)	Chi square	DR2 and DR3 removed		
				SLE (n = 340 alleles)	Controls (n = 1,405 alleles)	Chi square
	a			b		
DR1	6.5	11.3	12.7*	13.7	15.7	0.7
DR2	25.6	15.5	35.0*	—	—	—
DR3	26.7	12.5	76.9*	—	—	—
DR4	8.0	13.2	12.7*	16.9	18.3	0.3
DR5	8.4	14.1	15.0*	17.5	19.5	0.6
DR6	10.5	14.5	6.7	22.1	20.1	0.5
DR7	10.5	11.8	0.8	22.1	16.4	5.7 [‡]
DR8	2.4	2.8	0.1	5.1	3.8	0.8
DR9	0.4	1.5	4.3	0.9	2.1	1.6
DR10	0.7	1.6	2.3	1.5	2.2	0.3

After removal of DR3 only, chi square for RPE of DR2 equals 68.7 (35.0 versus 17.7% alleles); after removal of DR2 only, chi square for RPE of DR3 equals 111.6 (36.0 versus 14.8% alleles). * $P < 0.0005$; [‡] $P = 0.02$ (not significant after Bonferonni's correction for 10 antigens tested).

Table II. C4 Allele Frequencies (Percent) in 396 SLE Patients and Controls (Controls from [25])

C4 allele	SLE (n = 792)	Controls (n = 408)	P
C4AQ0	29	12	< 10 ⁻⁶
C4A1	0	1	NS
C4A2	3	7	0.0006
C4A3	61	66	NS
C4A4	4	8	0.01
C4A5	1	1	NS
C4A6	2	4	NS
C4BQ0	10	14	NS
C4B1	80	73	0.009
C4B2	10	9	NS
C4B3	0.5	2	NS

type frequency difference method in 162 SLE families, in which B-DR haplotypes were determined by segregation analysis and the frequencies of haplotypes transmitted to the patients are compared to nontransmitted haplotypes (33).

Do HLA-B7, HLA-B8, HLA-DR3, and HLA-DR2 constitute risk factors for SLE independently of the two haplotypes B7-DR2 and B8-DR3? The comparison of the haplotype and gene frequencies of 174 SLE patients of the family study as determined by direct allele counting revealed a significantly increased frequency of HLA-DR2 independent of the haplotype B7-DR2 in SLE patients versus non-SLE haplotypes and external controls (Table III). On the contrary, the frequency of HLA-B7 was found to be decreased in SLE outside the haplotype B7-DR2 (Table III). The numbers of patients with only DR3 without B8 and with only B8 without DR3 are small and no significant differences to controls were observed (Table IV).

Since the haplotype B8-DR3 carries a C4A deletion, we examined the prevalence of C4 deletions in SLE and controls. Table V shows the comparison of C4Q0 and C4 deletion frequencies of 155 SLE patients, as compared to the corresponding frequencies in 155 non-SLE haplotypes. As expected, C4A deletions were significantly increased, but not C4B deletions, C4B silent alleles, and C4A silent alleles (i.e., structurally intact C4 genes without detectable C4 gene product) (37). All C4B deletions were of the 21-OHA/C4B type, except for one C4B/21-OHB deletion associated with the haplotype B14-DR2.

Table VI shows that the majority (85 of 97; 87.6%) of C4A deletions in SLE patients and healthy haplotypes appear with the haplotype B8-DR3, and that all other C4A deletions appear with haplotypes carrying either HLA-B8 or HLA-DR3. Of all 86 B8-DR3 haplotypes examined, all but one carried the C4A deletion. It is interesting to note that of all C4AQ0 alleles appearing with B8 or DR3, the majority (75%) was due to C4A deletions.

Those C4AQ0 alleles without B8 or DR3, which usually are nonexpressed ("silent") C4A alleles, were not increased in SLE patients over non-SLE haplotypes or controls (Table IV). Many of the nondeleted C4AQ0 alleles (~ 40%) were found with the haplotype B60/61-DR6, thus confirming previous observations (27, 37). Other haplotypes observed with C4AQ0 silent alleles were B44-DR7, B7-DR6, B35-DR1, and B62-DR4. As shown in Table IV, the frequency of the haplotype B60/61-C4AQ0-DR6 is not increased in SLE.

In no instance was a C4AQ0 allele detected with the B7-DR2 haplotype. The B7-DR2 haplotype shows a strong linkage disequilibrium with the C4A3-C4B1 complotype (33, 38), as was the case in our sample (data not shown).

Finally, we examined the question whether heterozygous or homozygous combinations of the two risk haplotypes were evenly distributed according to the Hardy-Weinberg equilibrium, or whether they deviated from expected frequencies. As depicted in Table VII, the observed homozygous and heterozygous haplotype combinations in 174 SLE families did not differ from the expected values, as calculated from the haplotype frequencies. This result is in accordance with our previous observation that all homozygous and heterozygous DR combinations in SLE patients do not differ from the expected distributions (39).

Since some authors (40, 41) have postulated that homozygosity for C4AQ0 is a risk factor for SLE, we compared the observed frequencies of C4AQ0 homozygotes with the expected values on the basis of the observed C4AQ0 allele frequencies. The observed frequencies for C4AQ0 homozygotes and homozygotes for C4A deletions did not exceed expected values (Table VIII).

Discussion

So far it is unknown which of the MHC genes cause the association with SLE. Interpretation of disease associations with particular MHC alleles is complicated by the fact that strong linkage

Table III. Frequencies of the HLA-B7-DR2 Haplotype and Its Alleles in SLE and Controls

Haplotype	SLE		Controls		P values		
	174 SLE patients (n = 348)	Non-SLE haplotypes (n = 265)	German controls (n = 975)	European controls (n = 1,900)	p1	p2	p3
All B7	15.2	10.9	13.9	11.2	(0.15) NS	(0.62) NS	0.04
All DR2	25.6	14.7	15.6	15.8	0.0015	5 × 10 ⁻⁵	1.3 × 10 ⁻⁵
B7-DR2	10.9	5.3	7.0	5.4	0.02	0.03	0.0002
DR2 without B7	14.7	9.4	8.6	10.4	(0.07) NS	0.002	0.03
B7 without DR2	4.3	5.6	6.9	5.8	NS	NS	NS

n, number of haplotypes; p1, SLE versus non-SLE haplotypes; p2, SLE versus German controls (33); p3, SLE versus European controls (30).

Table IV. MHC Haplotypes and C4AQ0 Alleles in SLE and Controls (Percent)

	SLE		Controls	
	174 SLE patients (n = 348)	Non-SLE haplotypes (n = 265)	German controls (n = 975)	European controls (n = 1,900)
All C4AQ0	30.2*	17.0	12.0*	12.6
B8-C4AQ0-DR3	22.4*	8.3 [‡]	7.8	7.3
B8—all C4A—DRX	3.4	2.6	3.3	2.4
B8—C4AX—DRX	1.7	1.5	na	na
B8—C4AQ0—DRX	1.7	1.1	na	na
DR3—all C4A—BX	5.4	2.6	4.7	4.8
DR3—C4AX—BX	3.1	2.3	na	na
DR3—C4AQ0—BX	2.3	0.4	na	na
B8 or DR3—all C4A	8.9	5.3	8.0	7.1
B8 or DR3—C4AX	4.8	3.8	na	na
B8 or DR3—C4AQ0	4.0	1.5	na	na
C4AQ0 without B8 or DR3	4.0	6.8	na	na
All C4AQ0 without B8 and DR3	8.0	8.3	na	5.3
B60/61-C4AQ0-DR6	1.7	1.9	na	na

C4AX; all C4A but C4AQ0; BX, all HLA-B but B8; DRX, all HLA-DR but DR3; where not indicated, $P > 0.05$). * $P < 10^{-3}$, for all three control groups; [‡] from (25), $n = 408$ alleles; [§] without one B8—C4AX—DR3 haplotype; ^{||} na, not available.

disequilibria among different genes located in the MHC do not allow easy assessment of the risks associated with individual MHC alleles (38). It is therefore difficult to discern which components of a MHC haplotype are primarily responsible for susceptibility to SLE, and which are “innocent bystanders” due to linkage disequilibrium.

Our multicenter study confirmed the increase of B8-DR3 haplotypes in SLE previously reported (42–44) but also identified that B7-DR2 haplotypes are increased in Caucasian SLE patients. This is the first time that this haplotype has been found to be a risk factor for SLE, but the results are in accord with the previous findings of some groups reporting an increase of DR2 in SLE (13–15). Therefore, one or two susceptibility genes for SLE are present on those two haplotypes. Interestingly, DR2 is also increased independently of the B7-DR2 haplotype whereas B7 is not, which explains why B7 has not been

Table V. C4 Deletions and Silent Alleles in C4Q0 Alleles of SLE Patients and Non-SLE Haplotypes (Percent)

	SLE	Non-SLE	P
	310 haplotypes	155 haplotypes	
All C4AQ0	30.0	18.1	$< 10^{-6}$
C4A deletion	24.5	12.9	$< 10^{-6}$
C4A silent	5.5	5.2	NS
All C4BQ0	9.0	12.9	NS
C4B deletion	3.9	5.2	NS
C4B silent	5.1	7.8	NS

Table VI. C4A Deletions in C4AQ0 Haplotypes (Percent)

Haplotype	SLE haplotypes		Non-SLE haplotypes		All haplotypes	
	(n = 310)	%	(n = 155)	%	(n = 465)	%
B8-C4AQ0-DR3	(69/69)	100	(16/16)	100	(85/85)	100
B8-C4AQ0-x	(3/4)	75	(2/2)	100	(5/6)	83
y-C4AQ0-DR3	(6/9)	66	(1/1)	100	(7/10)	70
y-C4AQ0-x	(0/11)	0	(0/8)	0	(0/19)	0
Total	(78/93)	89	(19/27)	70	(97/120)	81

x, any DR antigen except DR3; y, any B antigen except B8.

found to be increased in SLE (36). This indicates that the susceptibility gene on the haplotype B7-DR2 has to be located closer to the DR locus than to the B locus. Whether the gene is DR2 itself or a gene in strong linkage to DR2 (for instance DQ) cannot be decided. DNA analysis of the DR2-positive haplotypes from the SLE patients of this study has shown a strong preponderance of the haplotypes DRB1*1501, DQA1*0102, and DQB1*0602 (Albert, E., personal communication) which is in accord with a previous report on 78 North American Caucasian SLE patients (45). However, in 14 Asian, Polynesian, and Filipino SLE patients with DR2, the allele DQB1*AZH (DQw5) was preponderant (45). It will be important to clarify whether the HLA-DR2 specificities which are increased in Chinese (46) and Japanese (47) SLE patients are of the same subtype as those found in Caucasian SLE.

The haplotype B18-DR2, which is in strong linkage disequilibrium with the C2Q0 allele (48), does not contribute significantly to the increase of DR2 in our SLE patients (33). The haplotype B17-DR7, which recently was found to be increased in SLE in one small study (42) but not in others (43, 44) was not increased in our SLE patients. Although the initial analysis of the DR gene frequencies resulted in significant negative associations of DR1, DR4, and DR5 with SLE, this phenomenon must be interpreted as a “steal effect” of the DR2 and DR3 alleles, because our calculation of relative predispositional effects (35) for the DR antigens showed that after the exclusion of DR2 and DR3 no other DR antigen was positively or negatively associated with SLE in a significant way. In some autoimmune diseases, like diabetes mellitus (49), effects of transcomplementation of MHC alleles have been ascertained, but this is not the case in SLE, as the frequencies of the B7-DR2 and B8-DR3 homozygotes and of the B7-DR2/B8-DR3 heterozygotes are not increased over the values expected from the allele frequencies.

Table VII. Heterozygous and Homozygous Combinations of Risk Haplotypes in 174 SLE Patients from a Family Study

	Percent observed	Percent expected
B8-DR3 homozygotes	4.0	5.0
B7-DR2 homozygotes	0.6	1.2
B7-DR2/B8-DR3 heterozygotes	4.0	4.9
Others	91.4	88.9

chi square = 0.76; not significant.

Table VIII. C4AQ0 Homozygosity in SLE

C4AQ0 homozygotes	Frequency			
	Observed	Expected		
		%	%	%
In 174 SLE patients (from the family study)	(n = 15)	8.6	(0.308) ²	9.5
In all 343 SLE patients	(n = 27)	7.8	(0.296) ²	8.8
Homozygous C4A deletions				
In 155 SLE patients (Examined by TaqI RFLP and C4 phenotyping)	(n = 9)	5.8	(0.245) ²	6.0

All $P > 0.2$, not significant; n = absolute numbers, allele frequencies in brackets.

Based on our results we do not favor the hypothesis that C4AQ0 alleles are the primary disease-associated genes for SLE, but that the risk is conveyed by the entire B8-C4AQ0-DR3 haplotype or a part of it. We find a frequency of the B8-DR3 haplotype of $\sim 21\%$, with a control frequency of 8% . The gene frequency of C4A deletions is $\sim 25\%$, with 4% of the C4A deletions being found on haplotypes with either B8 or DR3 alone. In contrast to C4A deletions, silent C4AQ0 alleles are not increased in our SLE patients; this is in agreement with our observation that the haplotype B60/B61-DR6, which is associated with a large proportion of the nonexpressed C4AQ0 genes, is not increased in SLE. Furthermore, C4AQ0 homozygotes are not increased in SLE over the expected values (as calculated from allele frequencies). In previous investigations it has been claimed that a dosage effect of C4AQ0 exists (40, 41), a finding that we have not confirmed.

Additional indirect evidence against a direct functional involvement of C4Q0 alleles in lupus is the fact that neither C4BQ0 nor C4B deletions are increased in SLE, which makes it necessary to find an explanation why a partial deficiency of C4A, but not of C4B, should predispose to SLE. Functional differences between C4A and C4B have been reported: for example, an increased capacity of C4A to solubilize immune complexes (50), but if this function was relevant for SLE, a dosage effect with an increased number of C4AQ0 homozygotes should have to be expected.

The common occurrence in various races of distinct alleles which are associated with disease susceptibility is often used to identify primary risk factors. An increase of C4AQ0, but not C4BQ0, alleles has been reported in blacks (51), in Japanese, and in Chinese (52). However, the C4AQ0 frequencies in Japanese and Chinese were based on phenotyping alone of much smaller groups than those tested here. In American blacks the increase in C4AQ0 is also due to an increase in C4A deletions which are phenotypically associated with DR2 and DR3 (51). Another recent report did not find any C4A deletions in Japanese SLE patients (53), which may be explained in part because the haplotype B8-DR3 does not exist in Japanese. To our knowledge, family studies in non-Caucasians have so far not been reported; they should be performed to identify those haplotypes carrying susceptibility genes for SLE in different races.

In conclusion, two MHC-linked genes predispose to SLE among Germans. One is probably located close to DR2 on the

haplotype B7-DR2, and one is located on the haplotype B8-DR3. These two genes have no additive effect on susceptibility when appearing in transcomplementation or in homozygosity. Various lines of evidence suggest that these genes are not complement Q0 alleles in our population.

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